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ORIGINAL ARTICLE

Melatonin triggers p53^{Ser} phosphorylation and prevents DNA damage accumulation

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Several epidemiological studies have shown that high levels of melatonin, an indolic hormone secreted mainly by the pineal gland, reduce the risks of developing cancer, thus suggesting that melatonin triggers the activation of tumor-suppressor pathways that lead to the prevention of malignant transformation. This paper illustrates that melatonin induces phosphorylation of p53 at Ser-15 inhibiting cell proliferation and preventing DNA damage accumulation of both normal and transformed cells. This activity requires p53 and promyelocytic leukemia (PML) expression and efficient phosphorylation of p53 at Ser-15 residue. Melatonin-induced p53 phosphorylation at Ser-15 residue does not require ataxia telangiectasia-mutated activity, whereas it is severely impaired upon chemical inhibition of p38 mitogen-activated protein kinase activity. By and large, these findings imply that the activation of the p53 tumor-suppressor pathway is a critical mediator of melatonin and its anticancer effects. Therefore, it provides molecular insights into increasing observational evidence for the role that melatonin has in cancer prevention.
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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a mammal pineal hormone that is secreted and subtly regulated by a circadian pacemaker located in the central nervous system. This regulation occurs at the hypothalamic level, which receives dark/light signals from the retina. Melatonin is mainly secreted at night and its synthesis is controlled by a set of enzymes. These enzymes are inhibited by the presence of sunlight and become more active in the dark. It has been reported that a reduction in melatonin concentration may be correlated with an increase in cancer occurrence. In fact, a number of epidemiological studies have not only shown that light exposure during night-shift work suppresses melatonin production but also that night work has been associated with an increased cancer risk. Several studies have evaluated the comparison of light exposure in night-shift workers with those of day-shift workers and its correlation with various types of cancers. To further support this association, studies conducted in blind women have reported an inverse relationship between the incidence of breast cancer and the degree of sightedness, with a 40% lower risk of breast cancer (Kliukiene *et al*., 2001; Flynn-Evans *et al*., 2009). It is worthy to note that blind women produce higher levels of melatonin as their retina receives less light and therefore their pineal gland is less induced to reduce melatonin production. Two prospective control studies nested in larger cohorts have been recently published (Schernhammer *et al*., 2008, 2010). The first study, conducted within the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) cohort, tested the concentration of a melatonin metabolite, 6-sulfatoxymelatonin (aMT6s) in 178 postmenopausal women with incident breast cancer and in 710 matched controls (Schernhammer *et al*., 2010). The second case–control study was within the National Health Interview Survey (NHIS) cohort (Schernhammer *et al*., 2008). Both studies show a strong inverse association between breast cancer and overnight urinary melatonin levels of aMT6s. Overall, these findings may indicate that melatonin's anti-neoplastic actions are highly associated with triggering tumor-suppressor pathways. It has previously been reported that melatonin induces both p53 and p21^{waf1} protein expressions presumably highlighting its oncostatic actions (Mediavilla *et al*., 1999). The p53 tumor-suppressor gene is frequently mutated in human tumors. In normal cells, p53 is present in low concentrations, owing to continuous and rapid degradation through the ubiquitin–proteasome system (Haupt *et al*., 1997; Kubbutat *et al*., 1997). In response to a plethora of stressful conditions, most notably those that may lead to genomic damage, p53 becomes activated (Wang and Prives, 1995; Shieh *et al*., 1997). This is reflected in an increase, at times quite sharp, in the overall amounts of the p53 protein within the cell, as well as in various post-translational modifications that augment the biochemical properties of p53. In its activated state, p53 can drive various cell-fated changes,

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ranging from a transient growth arrest all the way to replicative senescence and apoptotic cell death (Unger *et al*., 1999; Oren, 2003).

In this paper, we show that melatonin activates p53 by promoting its accumulation and phosphorylation at Ser15. This pairs with a p53-dependent reduction of cell proliferation and the ability to form colonies. In fact, this effect is severely impaired in cells in which the p53 gene expression is selectively knocked down. We also demonstrate that melatonin prevents DNA damage accumulation in both normal and transformed cells. This effect requires p53, PML protein expression and efficient phosphorylation of p53 at Ser-15 residue. The latter is phosphorylated by ataxia telangiectasia-mutated (ATM) kinase in response to γ-irradiation, whereas p38 mitogen-activated protein kinase (MAPK) leads to phosphorylation of p53 in Ser-15 in response to ultraviolet irradiation (She *et al*., 2000). Interestingly, melatonin-induced p53 phosphorylation does not require ATM activity as it occurs in both ATM+/+ and ATM−/− human fibroblasts and upon caffeine-mediated inhibition of ATM kinase activity. Conversely, by using specific inhibitors (PD98059 and SB202190) of p38 MAPK activity, p53 phosphorylation at Ser-15 is abolished. As a result, melatonin-induced prevention of DNA damage is impaired. Overall, these findings emphasize that activation of the p53 tumor-suppressor pathway may be a critical mediator in melatonin's anticancer activity.

Results

Melatonin induces p53-dependent inhibition of cell proliferation

To ascertain whether melatonin triggers the activation of tumor-suppressor pathways, we sought to analyze the p53 response to melatonin treatment of different cancer cell lines. We found that melatonin treatment (1 μM) of MCF-7 and HCT116 cells for the indicated time points induces both p53 and p21 protein accumulation (Figures 1A and b). We also observed that melatonin triggers p53 phosphorylation at Ser-15 residue but not at Ser-9, Ser-37 and Ser-46 (Figures 1a and b, Supplementary Figure S1F and data not shown). This pairs with no modulation of p53 mRNA levels (data not shown). In addition, melatonin treatment slows cell proliferation (Figure 1c) and impairs the ability to form colonies (Figure 1d) of MCF-7 cells. Inhibition of cell growth also occurred in human fetal fibroblasts (HFF) normal primary cells (Halvorsen *et al*., 1999) (Supplementary Figure S1A). To understand whether melatonin's effects on cell growth are p53 dependent, we measured the growth of cells and their ability to form colonies in isogenic HCT116 (Figures 1e and f) and HCT116 p53−/− (Figures 1g and h) colon cancer cell lines. Inhibition of cell proliferation by melatonin is lost in HCT116 p53−/− cells (Figures 1g and h). We tested whether cell proliferation inhibition was due to perturbations in the cell cycle. Melatonin treatment induced a transient G2 arrest in MCF-7 cells (Figure 1i) and a transient G1 arrest, with a decrease in the S phase, in HCT116 cells (Figure 1l). These changes occurred within a few hours after melatonin addition and could not be detected later (Supplementary Figures S1B and C). We also asked whether melatonin caused either apoptosis or senescence. On the basis of cell growth curves, we performed annexin V staining on MCF-7 cells treated with melatonin for 72 h and on HCT116 cells at 48 h after treatment (Supplementary Figure S1D). Neither of the cell lines showed apoptosis after melatonin treatment. In addition, we tested whether melatonin triggered senescence by senescence-associated β-galactoside staining by using the same experimental setup. As a positive control, we used WRN−/− fibroblasts, which undergo spontaneous senescence (Faragher *et al*., 1993). We did not observe any variation in the number of senescent MCF-7 or HCT116 cells upon melatonin treatment (Supplementary Figure S1E). We speculate that a transient delay of the cell cycle induced within a few hours from adding melatonin would result in a decrease in cell number.

Melatonin reduces genetic alterations after exposure to DNA-damaging agents

It has been previously shown that p53 phosphorylation at Ser-15 is associated with DNA damage response. This prompted us to investigate whether melatonin may trigger its cancer prevention action by activating p53. Thus, by preventing the accumulation of mutations in response to DNA insults, active p53 maintains the integrity of the genome. To this end, we used the random amplified polymorphic DNA (RAPD) technique that allows the rapid and easy detection of genome-wide genetic alterations (Xian *et al*., 2005). First, we analyzed whether continuous treatment (for 5 days) with melatonin provokes DNA mutations in both MCF-7 and MCF-10A breast cell lines by PCR amplifying the genomic DNA with seven different primers. No mutations were observed in both cell lines upon melatonin treatment (Figure 2a). To assess the genetic alterations induced by DNA-damaging treatment, we treated MCF-7 and MCF-10A breast cell lines with and without sublethal doses of chemotherapeutic drugs for 10 days both in the absence and in the presence of melatonin. As shown in Figure 2b, treatment with the DNA-damaging agent Cisplatin (CDDP, lane 2) led to a change in the amplification pattern, indicating a generation of mutations. Cells co-treated with melatonin and CDDP (lane 3) did not show any changes (or very modest changes) in the band pattern as compared with untreated cells (lane 1), thereby suggesting that melatonin prevents DNA mutations (Figure 2b). Subsequently, we hypothesized that melatonin may prevent mutations by inducing DNA repair. To test this hypothesis, we investigated whether melatonin could affect DNA fragmentation by performing comet assays. We treated MCF-10A (Figure 2c) and MCF-7 (Figure 2d) cells, first with melatonin for 4 h and then by adding an alkylating agent (CDDP) or antimetabolite (FU) or a topoisome-

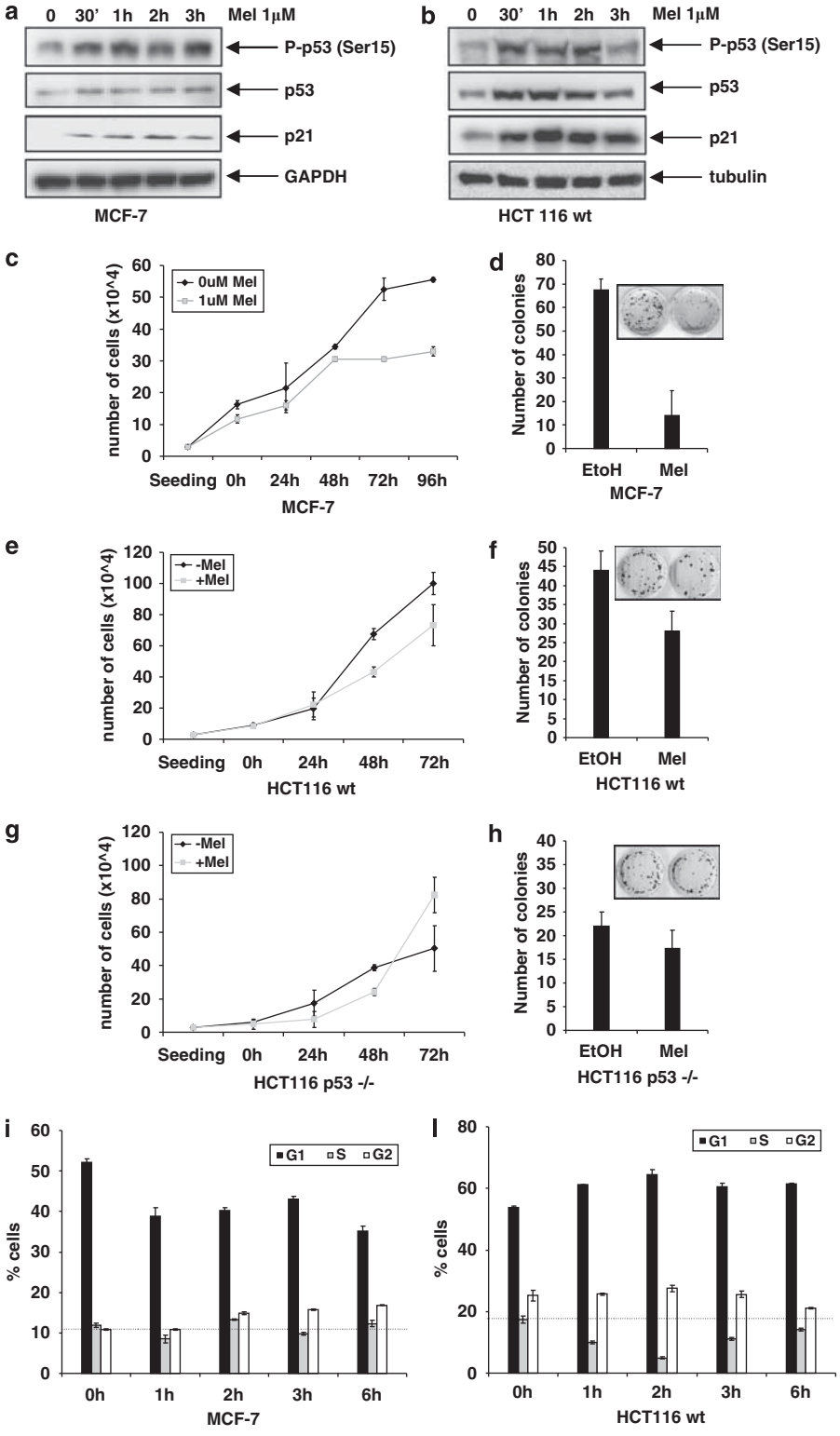

Figure 1 Melatonin activates p53. (**a**, **b**) MCF-7 (panel **a**) and HCT116 (panel **b**) cells were treated with 1 μM melatonin for the indicated time; cells were lysed and protein extracts underwent immunoblot with the indicated antibodies. (**c**, **e** and **g**) MCF-7 (panel **c**), HCT116 (panel **e**) and HCT116 p53−/− (panel **g**) cells were grown for the indicated time in the absence and in the presence of 1 μM melatonin; cells were counted and the cell number is shown on the graph. (**d**, **f** and **h**) MCF-7 (panel **d**), HCT116 (panel **f**) and HCT116 p53−/− (panel **h**) cells were seeded and grown for 15 days in the absence or presence of 1 μM melatonin; colonies were stained with crystal violet and counted. Colony number is shown. (**i**–**l**) MCF-7 (panel **i**) and HCT116 (panel **l**) cells were treated with melatonin for the indicated time and BrdU incorporation was performed. Cell-cycle distribution is shown.

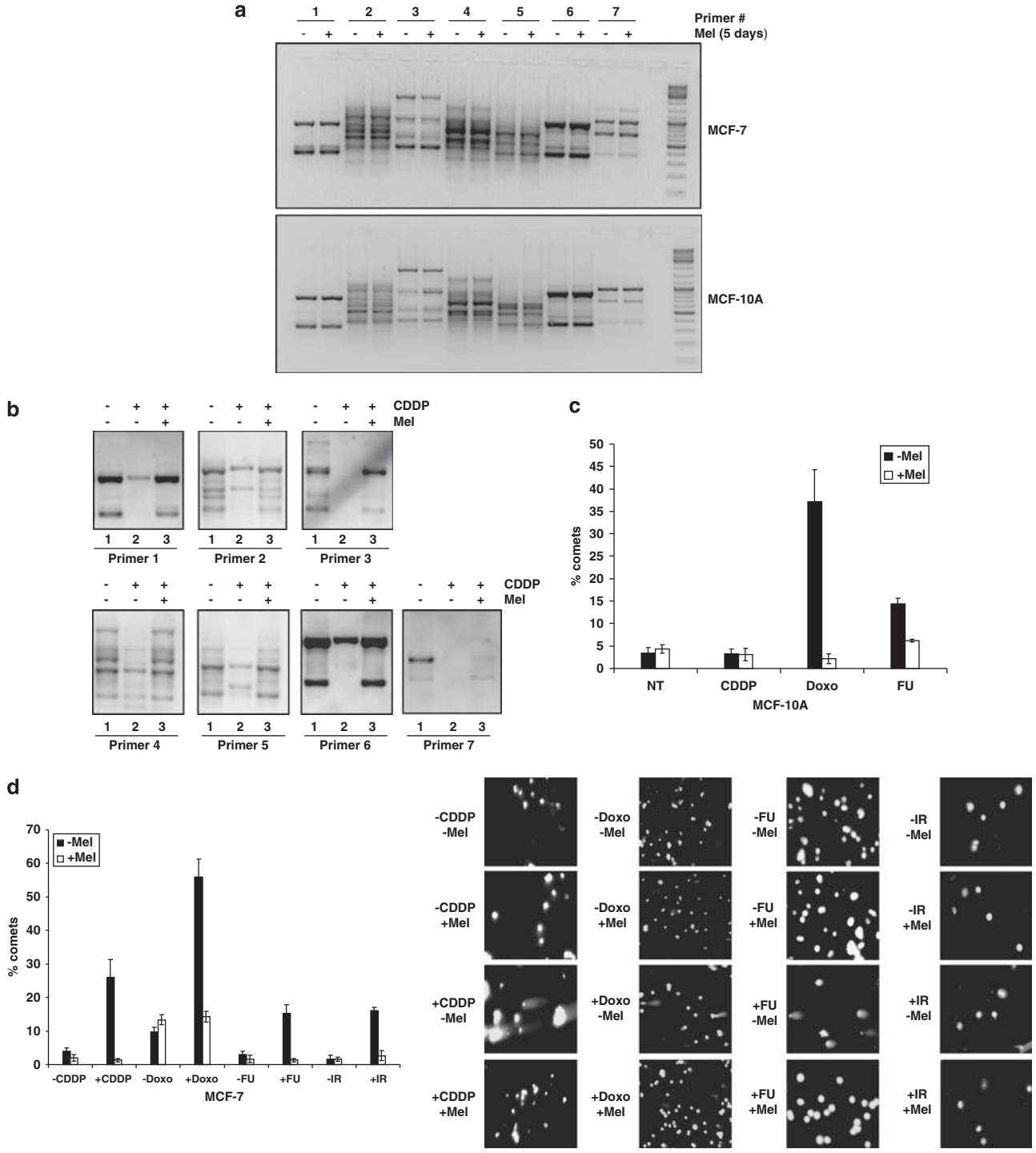

Figure 2 Melatonin prevents DNA mutations. (**a**) MCF-7 and MCF-10A cells were grown in the absence or in the presence of 1 μM Mel for 5 days; genomic DNA was extracted and RAPD analysis was performed. (**b**) MCF-7 cells were kept in culture for 10 days with the indicated chemotherapeutic agent in the absence or in the presence of melatonin. The presence of DNA mutations was assessed by RAPD analysis. Numbers indicate lanes. (**c**) MCF-10A cells were treated with 1 μM melatonin for 4 h and then the indicated chemotherapeutic agents were added for 22 h. Cells underwent comet assay. (**d**) MCF-7 cells were treated as described in panel **c**; γ-irradiation (10 Gγ) was performed 4 h after melatonin addition and cells were allowed to repair DNA for 3 h after irradiation. Sample pictures are shown in the right panel.

rase inhibitor (Doxo) for 22 h. In addition, we performed gamma-irradiation (IR, 10 Gγ) and comet assays 3 h after irradiation. Comet assays revealed that melatonin does not induce DNA damage *per se* and reduces the number of comets generated by chemotherapy and radiotherapy (Figures 2c and d). Overall, these findings show that melatonin prevents DNA damage by promoting DNA repair.

We asked whether melatonin-induced DNA repair may protect cancer cells against chemotherapeutic treatment. Therefore, we performed clonogenic assays pretreating cells with melatonin for 4 h and then adding either doxorubicin (Doxo) or cisplatin (CDDP) for 22 h. Despite promoting DNA repair, the presence of melatonin did not interfere nor impede with chemotherapy in impairing colony formation (Supplementary Figure S2). The same results were observed when testing cell proliferation (Supplementary Figures S3A–D).

Melatonin reduces DNA fragmentation through p53 and PML proteins

Given that we observed a p53-dependant reduction of cell proliferation by melatonin, we asked whether reducing DNA fragmentation would rely on p53 as well. To this end, we used both MCF-7, the p53 expression of which was selectively silenced, and isogenic HCT116 and HCT116 p53−/− cells. Melatonin reduced DNA fragmentation both in control siRNA-transfected MCF-7 cells and in HCT116 wild-type cells (Figures 3a and c), but failed to do so in both p53 siRNA-transfected MCF-7 and isogenic HCT116 p53−/− cells (Figures 3b and d). These findings showed that p53 protein expression was required to reduce DNA fragmentation upon melatonin treatment. These findings are further supported by experiments performed in p53+/+ and p53−/− mouse embryo fibroblasts (Lapi *et al*., 2008). Again, melatonin failed to reduce DNA fragmentation in mouse embryo fibroblast p53−/− (Figure 4b), whereas it efficiently did in their wild-type counterparts (Figure 4a). Growing evidence reports that p53 orchestrates DNA damage response and DNA repair by interacting with diverse cellular partners. Among them, PML is closely connected to p53 being both a p53 transcriptional target gene and a p53-interacting protein (Guo *et al*., 2000; Pearson and Pelicci, 2001; Bernardi *et al*., 2004). Therefore, we investigated the potential involvement of PML protein in cellular responses to melatonin. First, we examined PML protein levels upon melatonin treatment. We observed that melatonin induced PML accumulation (Figures 4c and d), similar to p53 (Figures 1A and b). We then performed comet assays in PML−/− mouse embryo fibroblast and found that PML deficiency impaired melatonin's capability to reduce DNA fragmentation (Figure 4e), suggesting that PML is required for this process. To ascertain whether PML has a direct role in melatonin-induced p53-dependent DNA repair, we tested whether PML may be involved in the phosphorylation of p53 after melatonin treatment. We silenced PML and then treated cells with melatonin

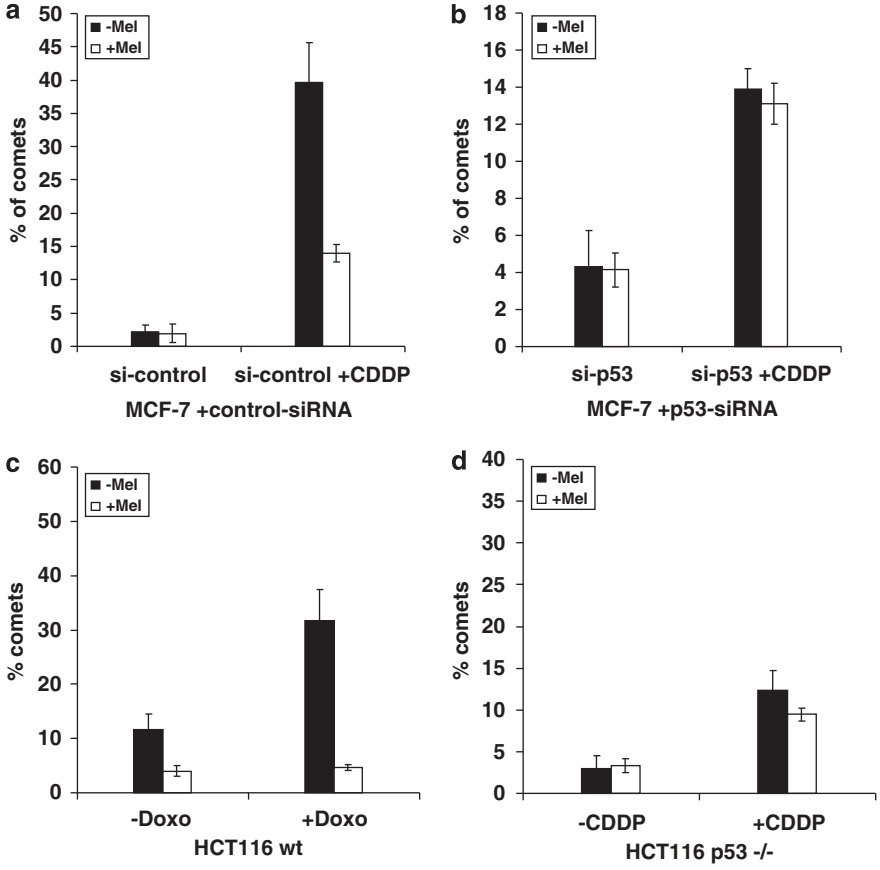

Figure 3 Melatonin inhibits DNA fragmentation in a p53-dependent manner. (**a**, **b**) MCF-7 cells were transfected with control siRNA (panel **a**) or p53 siRNA (panel **b**) and treated as described in Figure 2c. (**c**, **d**) HCT116 wt (panel **c**) and p53-/- (panel **d**) cells were treated as in panel **a**. Percentage of comets is shown.

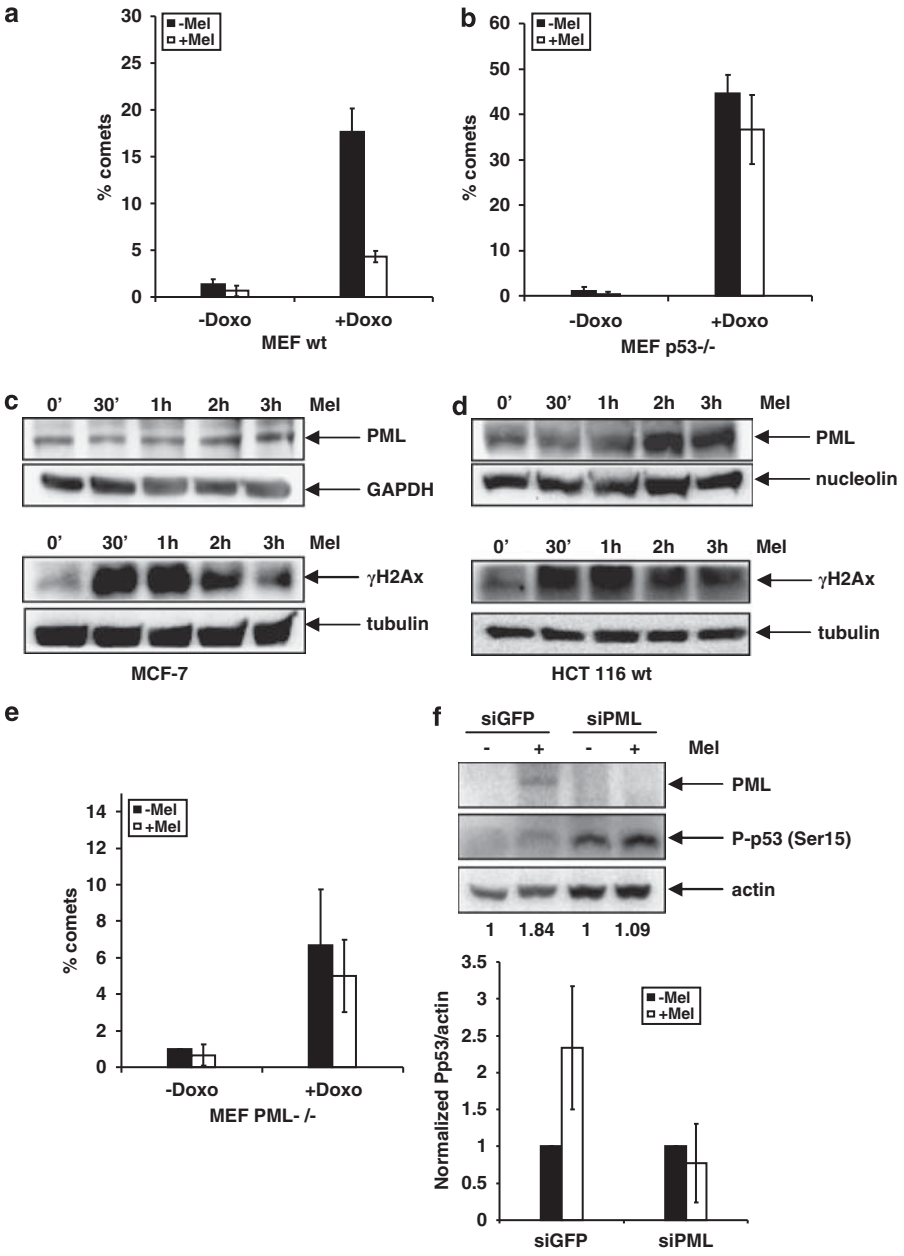

Figure 4 Melatonin inhibits DNA fragmentation in a PML-dependent manner. (**a**, **b** and **e**) MEF wt (panel **a**), p53−/− (panel **b**) and PML−/− (panel **e**) underwent comet assay. Percentage of comets is shown. (**c**, **d**) MCF-7 (panel **c**) and HCT116 (panel **d**) cells were treated with 1 μM melatonin for the indicated time and protein extracts underwent immunoblot with the indicated antibodies. (**f**) HCT116 cells were transfected with control siRNA and PML siRNA. Ninety-six hours after transfection, cells were treated with melatonin for 2 h. Cells underwent immunoblot with the indicated antibodies. Numbers indicated densitometric quantification of the ratio phospho-p53/actin. Densitometric quantification of phospho-p53/actin, normalized to untreated, for two independent experiments is shown in the graph.

for 2 h. Our findings showed that PML knockdown impaired phosphorylation of p53 induced by melatonin (Figure 4f).

We also investigated whether melatonin could influence the expression of other DNA damage-related proteins that either cooperate with or regulate p53, such as ATM and H2AX (Banin *et al*., 1998; Fernandez-Capetillo *et al*., 2002; Nur *et al*., 2003; Shrivastav *et al*., 2008). The latter becomes phosphorylated upon DNA damage and acts as a sensing and repair molecule (Lukas and Bartek, 2009). Upon treating MCF-7 and HCT116 cells with melatonin for the indicated time, we checked the protein levels of H2AX phosphorylated in Ser-139 (γH2AX). Interestingly, we observed an increase in γH2AX protein levels in both MCF-7 (Figure 4c) and HCT116 cell lines (Figure 4d). We ascertained that melatonin-induced H2AX phosphorylation was not totally due to an increase in the total level of H2AX

protein by western blot (Supplementary Figures S4A and C) and densitometric analysis (Supplementary Figures S4B and D).

To determine whether DNA machinery was triggered after treatment with melatonin and CDDP alone or in combination, we performed immunofluorescent staining of γH2AX for up to 6 h after treatment. Melatonin and CDDP alone, as well as in combination, induced γH2AX foci formation within 1 h of treatment in both MCF-7 (Supplementary Figure S4E) and HCT116 (Supplementary Figure S4F).

Melatonin-induced p53 phosphorylation is mediated by p38 MAPK but not by ATM kinase

Given that ATM is known as the principal regulator of p53 function upon DNA damage (Banin *et al*., 1998), there was a need to study the role of ATM in melatonin-induced p53 phosphorylation. To this purpose, we treated ATM-deficient and ATM-reconstituted cells with melatonin and monitored p53 phosphorylation. Interestingly, melatonin treatment induced p53 phosphorylation in both ATM-deficient cells and ATM-reconstituted cells (Figure 5a). Furthermore, to chemically inhibit ATM, we treated MCF-7 cells with caffeine for 2 h and then added melatonin for 2 h. Caffeine did not impair p53 phosphorylation after melatonin treatment (Figure 5b and Supplementary Figure S6). In addition, we performed comet assays in ATM-deficient cells and ATM-reconstituted cells. Melatonin reduced DNA fragmentation even in the absence of ATM (Figure 5c), thus providing further support to our finding that ATM is not required for the activation of p53 by melatonin. When the DNA repair machinery is activated, ATM is phosphorylated in Ser-1881 (Shiloh, 2003). This prompted us to investigate whether melatonin had any effect on ATM phosphorylation. We performed immunofluorescent staining of phosphorylated ATM in MCF-7 and HCT116 cells treated with either melatonin or CDDP alone or in combination. CDDP alone induced phosphorylation of ATM, whereas melatonin failed to do so (Supplementary Figure S5). The combination of the two compounds did not lead to any further increase in phosphorylated ATM (Supplementary Figures S5A and B).

Evidence shows that p38 MAPK phosphorylates p53 at Ser-15 residue (She *et al*., 2000; Kim and Yoo, 2010). To assess whether melatonin-induced p53 phosphorylation occurs through p38 kinase activity, we first checked p38 MAPK expression levels after adding melatonin. We observed that melatonin promoted p38 MAPK protein accumulation (Figure 6a). It is worth mentioning that the chemical inhibition of p38 MAPK activity by using PD98059 (a MEK1 inhibitor) and SB202190 (a p38α and p38β inhibitor) severely impaired melatonin-induced p53 phosphorylation at Ser-15 in MCF-7 cells (Figure 6b). Therefore, we hypothesized that p53-mediated reduction of DNA fragmentation by melatonin could also be affected. To this purpose, we performed comet assays in MCF-7 cells treated with either ethanol or PD. PD impaired melatonin's ability to reduce DNA fragmentation caused by CDDP treatment (Figure 6c).

Overall, these findings illustrate that to reduce DNA damage accumulation by melatonin, efficient phosphorylation of p53 by p38 MAPK is required.

As H2AX can be phosphorylated through the p38 pathway upon DNA damage (Chiu *et al*., 2008), we also tested whether chemical inhibition of p38 would result in impaired phosphorylation of H2AX upon melatonin treatment. Treatment with PD and SB inhibitors impaired melatonin-induced H2AX phosphorylation (Figure 6b), similar to the results observed for p53.

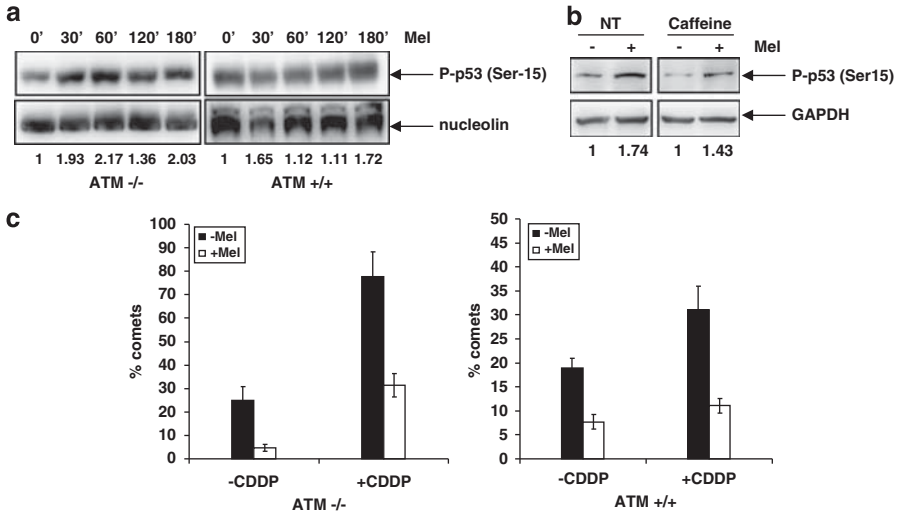

Figure 5 ATM is not required for melatonin-induced p53 activation. (**a**) ATM−/− and ATM-reconstituted cells were treated with melatonin for the indicated time; protein extracts were subjected to immunoblot with the indicated antibodies. Numbers indicated densitometric quantification of the ratio phospho-p53/nucleolin. (**b**) MCF-7 cells were either pretreated or not with 10 mM caffeine for 2 h and then either treated or not with 1 μM melatonin for additional 2 h; protein extracts underwent immunoblot with the indicated antibodies and densitometric quantification is shown. (**c**) ATM−/− (left panel) and ATM+/+ (right panel) cells underwent comet assay; percentage of comets is shown.

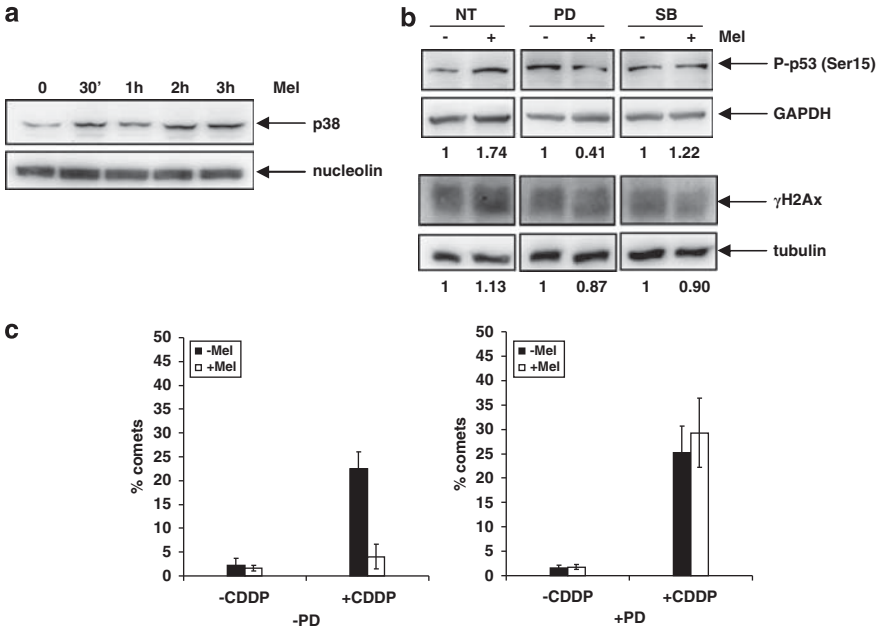

Figure 6 p38MAPK activity is required for melatonin-induced p53 phosphorylation. (**a**) MCF-7 cells were treated with melatonin for the indicated time; protein extracts underwent immunoblot with the indicated antibodies. (**b**) MCF-7 cells were either pretreated or not with 5 μM PD98059 or 5 μM SB202190 for 2 h and then either treated or not with 1 μM melatonin for an additional 2 h; protein extracts underwent immunoblot with the indicated antibodies and signal quantification is shown. (**c**) Cells untreated (left panel) and treated with PD98059 (right panel) underwent comet assay; percentage of comets is shown.

Discussion

There is widespread belief that finding novel therapeutic and preventive approaches through basic research are the key elements towards fighting cancer successfully.

Epidemiological studies have shown that melatonin decreases the risk of developing breast and other kinds of cancer (Kliukiene *et al*., 2001; Kloog *et al*., 2008; Schernhammer *et al*., 2008, 2009, 2009, 2010; Alpert *et al*., 2009). However, very little is known about the molecular mechanisms by which melatonin accomplishes its anticancer effects. This may occur through downregulation of aberrant oncogenic activities or activation of tumor-suppressor pathways. This paper provides strong evidence how melatonin exerts its oncostatic effects by activating the p53 tumor-suppressor pathway. This correlates with previous observations showing that melatonin induces p53 and p21 proteins, leading to inhibition of cell proliferation (Mediavilla *et al*., 1999). p53 is a master regulator that elicits anticancer effects ranging from growth arrest and apoptosis to senescence and DNA repair. These effects are achieved by p53 through a combination of transcriptional and post-translational events. p53 is a transcription factor that regulates the expression of a large cohort of target genes. Growing evidence indicates that the transcriptional activity of p53 is tightly regulated by a complex network of protein–protein interactions. The outcome of this is either p53 accumulation or destabilization, resulting in potentiating or impairing its tumor-suppressor activities. Melatonin treatment induces the accumulation of heavily phosphorylated p53 protein (Kim and Yoo, 2010). This occurs at a specific p53 residue (Ser-15) (Kim and Yoo, 2010) that has been shown to have a critical role in orchestrating p53-reponse upon DNA-damaging agents (Lakin and Jackson, 1999; Helton and Chen, 2007). In response to DNA damage, different kinases, including ATM and p38 MAPK, phosphorylate p53 at Ser-15 residue. We demonstrate that melatonin-induced p53 phosphorylation requires p38 MAPK activity (Figure 6), but not ATM (Figure 5). It is crucial to mention that melatonin treatment does not provoke any DNA damage in both immortalized and transformed cells. Despite this, melatonin induces the expression of the DNA damage-sensing and repair proteins γH2AX and PML (Figures 4c and d), which are known interactors of p53 (Carbone *et al*., 2002). ATM and ATR phosphorylate H2AX when double-strand breaks occur. However, in the absence of double-strand breaks (that is, when single-strand breaks occur instead) or in cells lacking ATM, other kinases can phosphorylate H2AX (Kinner *et al*., 2008; Shrivastav *et al*., 2008). p38 MAPK is one of those kinases (Chiu *et al*., 2008). This actually happens in cells treated with melatonin, in which γH2AX increases without ATM phosphorylation, given the absence of double-strand breaks (Supplementary Figures S4 and S5). Further support is provided by the impairment of H2AX phosphorylation by melatonin upon chemical inhibition of p38 MAPK (Figure 6b). Owing to its radical scavenger properties, melatonin can prevent DNA fragmentation caused by free radical-generating chemicals, such as H_2O_2 (Sliwinski *et al*., 2007). Here, we show that melatonin induces DNA repair proteins. This results in DNA damage reduction after various types of DNA insults. This effect is achieved by assembling a p53-mediated

DNA repair response that significantly reduces the accumulation of mutations (Figure 2b). In addition, we demonstrated that p53 activation by melatonin does not lead to apoptosis induction or p53-mediated senescence (Supplementary Figure S1). Most importantly, this does not pair with p53-mediated pro-survival effects, as melatonin does not counteract apoptosis induced by chemotherapy (Supplementary Figures S2 and S3A–D). One study demonstrated that 1 mM melatonin induces p53 phosphorylation at Ser-15 and apoptosis in androgen-sensitive LNCaP prostate cancer cells (Kim and Yoo, 2010). The concentration of melatonin used in this study is far higher than both physiological levels (1 mM vs 1 nM) and levels reached when melatonin is used to treat sleep disorders or as an adjuvant for anticancer therapy (Vakkuri *et al*., 1985; Kane *et al*., 1994; Markantonis *et al*., 2008), in which the highest concentration is 0.5 μM.

A previous report by Xi *et al*. (2000) showed that physiological concentrations of melatonin (for example, 0.5–5 nM) are able to reduce cell proliferation in LNCaP cells. A report from Sainz *et al*. (2005) showed that melatonin (0.5–2 mM for 6 days) induced marked G1 arrest and S-phase reduction, but no change in cell viability was observed. These data pair with our reported findings.

Owing to air, soil and water pollution, we are continuously exposed to DNA insults. Under physiological conditions, DNA insults determine DNA damage that can be largely mended by the cell's DNA repair machinery. Inevitably, a number of errors occurring in the DNA will not be repaired efficiently and cells will accumulate mutations. On the other hand, in the event of a DNA insult occurring, particularly when a certain amount of proteins is involved in the DNA repair, the cell is ready to repair DNA lesions and thus a lower number of mutations are consequently generated. It is reasonable to speculate that melatonin anticancer activity may have a role in safeguarding DNA from insults that might otherwise cause genetic alterations. This occurs, at least in part, through a specific cascade of events including p38-mediated phosphorylation, activation of p53 and consequently the induction of DNA repair proteins (Figure 7). An increase in the DNA repair machinery by melatonin might: (1) prevent carcinogenesis in healthy subjects, making it suitable to use in primary chemoprevention; (2) prevent accumulation of additional mutations in pre-cancerous lesions, thereby preventing carcinogenesis in high-risk patients and therefore be used in secondary chemoprevention; (3) prevent mutations in cancer cells, reducing the risk of developing a more aggressive phenotype (for example, invasiveness and metastatization) and consequently avoid giving the worst prognosis to cancer patients (tertiary chemoprevention); (4) limit the bystander effects induced by anticancer therapy in healthy tissues, thus maintaining genome stability and preventing the formation of second malignant neoplasms. In order for this to be possible, further study is required on the molecular chemoprevention targets triggered by melatonin.

Materials and methods

Cell culture and transfection

HCT116, MCF-7, mouse embryo fibroblast, ATM−/− and ATM +/+ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA). In addition, ATM−/− and ATM +/+ cells were cultured in the presence of 100 μg/ml hygromicin B (Sigma, St Louis, MO, USA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium F-12 with Glutamax, which is supplemented with 5% horse serum (Invitrogen), 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. All cell lines were grown at 37 °C in 5% CO_2. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

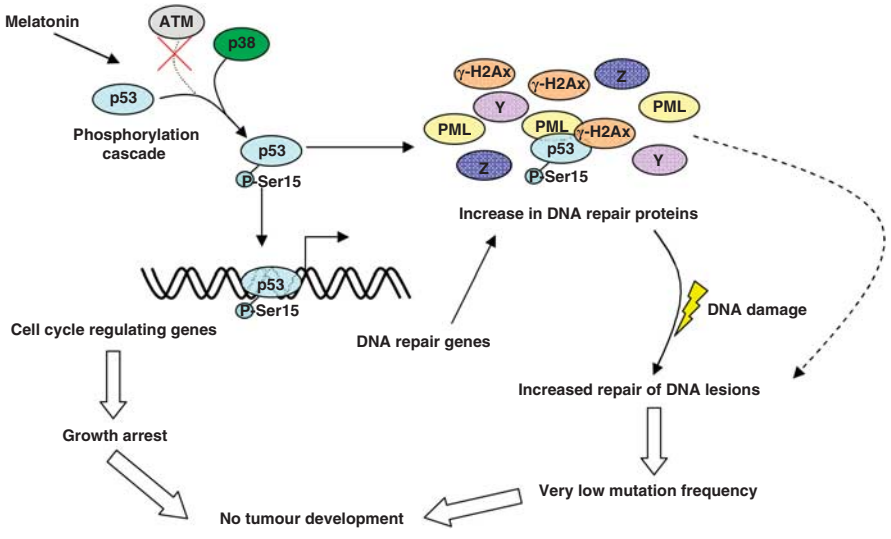

Figure 7 Model depicting melatonin's action. Melatonin treatment induces a p38-dependent phosphorylation cascade that activates p53 and other repair proteins. This translates in an increased ability of cells to detect DNA damage and repair it. This results in a lower mutation frequency.

MCF-7 stable cell lines were obtained as follows: cells were transfected with pRS-sh-LacZ and pRS-sh-p53 and resistant cells were selected by treatment with 1 μg/ml Puromycin (P8833, Sigma) for 5 days.

Reagents
Melatonin (M5250), caffeine (C0750), PD98059 (P215), SB202190 (S7067) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Growth curves and colony-forming assays
For growth curves, 7×10^3 cells/well were seeded in 24-well dishes. Cells were harvested at the indicated times by trypsin detachment and counted either manually (Figure 1 and Supplementary Figure S1A) or automatically using a Guava EasyCyte 8HT flow cytometer (Millipore, Billerica, MA, USA). In the latter case, cell concentration (cells/ml) is shown on the *Y* axis.

With regard to colony-forming assays, 5×10^2 cells were seeded in 35-mm dishes and grown for 15 days. Cells were stained with crystal violet and colonies counted.

To maintain melatonin levels (Roy *et al*., 2001), the medium was changed every 48 h in both experiments.

Western blots
Total protein extracts were prepared by lysing cells in 8 M urea. For nuclear extract preparation, cells were lysed in hypotonic buffer (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate and protease inhibitors), nuclei were pelleted at 500 rcf for 10 min and lysed in nuclei lysis buffer (50 mM Tris–Cl, pH 8.0, 10 mM EDTA, 1% SDS and protease inhibitors). Acid extracts (for total H2AX staining): cells were lysed in 1 volume TEN buffer (0.5% Triton in phosphate-buffered saline (PBS)) on ice for 10 min, centrifuged at 500 rcf for 10 min at 4 °C and washed once more with TEN buffer; nuclei were then lysed overnight at 4 °C in a half volume of 0.2 N HCl; lysates were buffered with 1/10 volume of 100 mM Tris, pH 8.0.

All protein extracts were quantified by Bradford assay and equal amounts were loaded onto SDS–PAGE, transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore) and underwent immunoblot with the indicated antibodies. Antibodies to p53 (sc-125, DO-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p53 Ser-15 (9284, Cell Signaling, Danvers, MA, USA), β-actin (A2288, AC-74, Sigma), p21 (2947, Cell Signaling), nucleolin (ab13541, 4E2, Abcam, Cambridge, UK), γH2AX (9718, 20E3, Cell Signaling), p38 (9212, Cell Signaling) and PML (sc-5621, H-238, Santa Cruz Biotechnology) were diluted in 5% bovine serum albumin/Tris-buffered salines/0.1% Tween-20. Secondary anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad (Hercules, CA, USA). Images were acquired using a VersaDoc MP instrument (Bio-Rad). Signal intensity was quantified using the Quantity One software (Bio-Rad) where indicated.

Immunofluorescence
In all, 2.5×10^4 cells were plated in a 8-well chamber slide (177402, Lab-Tek, Nunc, Roskilde, DK) and treated as indicated. Cells were fixed for 20 min with 4% paraformaldehyde in PBS and permeabilized for 25 min with 0.2% Triton X-100 in PBS. Cells were then incubated with the indicated primary antibodies for 1 h and subsequently with goat anti-rabbit Alexa Fluor 488 (A11034, Invitrogen) and goat anti-mouse Alexa Fluor 594 (A11032, Invitrogen). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Pictures were taken using × 63 magnification on an Axiovert 200 M microscope and Axiovision acquisition program (Zeiss, Thornwood, NY, USA).

RAPD analysis
In all, 100 ng of genomic DNA was amplified by PCR as described previously (Xian *et al*., 2005) with the following primers: #1: 5′-CCGGCTACGG-3′; #2: 5′-CAGGCCCTTC-3′; #3: 5′-AACGGTCACG-3′; #4: 5′-AGCTGCCGGG-3′; #5: 5′-AGGCATTCCC-3′; #6: 5′-GGTCTGAACC; #7: 5′-AAGGCTAACG-3′. PCR products were run onto a 2% agarose gel (Bio-Rad), stained with ethidium bromide (Sigma) and images were acquired using a VersaDoc MP instrument (Bio-Rad).

Comet assays
After treatment, cells were detached with trypsin and embedded in 1% low melting agarose (Sigma) in PBS and spread onto microscopy slides previously coated with 1% Agarose (Bio-Rad). Cells were lysed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 8 g/l NaOH, 1% Triton X-100, 10% dimethyl sulfoxide) for 1 h at room temperature and then run in running solution (300 mM NaOH, 1 mM EDTA, pH 13.0) for 30 min at 25 V and 250 mA. DNA was equilibrated with 0.4 M Tris pH 8.0 and slides were dried with methanol. DNA was stained with propidium iodide (Sigma) and pictures were taken using × 20 magnification on an Axiovert 200 M microscope and Axiovision acquisition program (Zeiss). At least 300 cells were scored for each slide.

Cell-cycle distribution
Cells were treated with melatonin for the indicated time and processed as described in the study by Ji *et al*. (2010). Samples were acquired using a Guava EasyCyte 8HT flow cytometer (Millipore). Cell-cycle distribution is shown.

BrdU incorporation
Cells were treated as indicated in the figures legend, labeled with 10 μM BrdU (5-bromo-2′-deoxyuridine) for 40 min. Cells were then detached with trypsin and stained using the '5-Bromo-2′-deoxy-uridine labeling and detection kit I' (11296736001, Roche, Basel, Switzerland) following the manufacturer's guidelines. Samples were acquired using a Guava EasyCyte 8HT flow cytometer (Millipore). Cell-cycle distribution is shown.

Annexin V staining
Cells were treated as indicated in the figures legend. Cells were detached with trypsin and stained using the 'Vybrant Apoptosis Assay Kit #2' (V13241, Invitrogen) following the manufacturer's recommendations. Samples were acquired using a Guava EasyCyte 8HT flow cytometer (Millipore). Percentage of Annexin V positive cells is shown.

Senescence-associated β-galactoside staining
Cells were treated as indicated in the figures legend. Cells were fixed with 3% paraformaldehyde for 5 min at room temperature, washed with PBS and stained overnight at 37 °C with senescence-associated β-galactoside staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal, pH 6.0).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)